REMARKS

In this Amendment, claims 37, 39, 40, 43, 49, 50 and 56 are amended, and claims 38, 41, 42, 44-48, and 51-55 are canceled. Thus, after entry of this Amendment, claims 37, 39, 40, 43, 49, 50 and 56 will be pending in the application.

The following amendments have been made to the claims.

Claim 40 has been amended to recite a "composition."

Claim 43 has been amended to recite contacting PBMCs with the peptide of SEQ ID NO: 188 "in vitro."

Claims 49 and 50 have been amended to more particularly recite the correlation between recognition of the peptide by CTLs and INF-γ production.

The claims have also been amended to exclude non-elected subject matter, and to clarify the claimed invention.

The specification has been amended to include sequence identifiers for all disclosed sequences encompassed by the rules, to indicate the sequence identifiers for the sequences disclosed in the Figures, and to inactivate hyperlinks.

Further, a Substitute Sequence Listing with a computer readable copy of the same, and the necessary Statement in support thereof, is being submitted herewith.

No new matter has been introduced.

Entry of this Amendment and the attached Substitute Sequence Listing is requested.

I. Compliance With Sequence Disclosure Rules

In response to the communication attached to the Office Action (form PTO-90C), and in response to the objections to the specification set forth at paragraph 4 of the Office Action, a Substitute Sequence Listing is being filed herewith, and the specification has been amended consistent with the same.

The Substitute Sequence Listing includes all sequences disclosed in this application that are encompassed by the sequence disclosure rules.

Further, amendments have been made to the specification to refer to the sequences disclosed in Figures 7a, 8b, 11, 13a and b, and on pages 61 and 70 of the application.

This application is believed to be in full compliance with the sequence disclosure rules.

II. Elected Subject Matter and Rejoinder

As indicated at item 2 of the Office Action, rejoinder of claims 43, 49, and 50, and designation of claim 56 as elected subject matter is appreciated.

Applicants have now canceled non-elected subject matter from the claims without prejudice.

Applicants reserve the right to file one or more divisional applications directed to non-elected subject matter.

III. Objections to the Specification and Claims

(1) At item 5 of the Office Action, the Examiner objects to the disclosure because it contains an embedded hyperlink and/or other form of browser-executable code at pages 18, 29, 56, and 57. The Examiner requires that these be deleted.

The internet addresses have been further amended so as to be inactive.

However, the Examiner's attention is respectfully directed to MPEP §608.1, which does not require deletion of the hyperlinks.

MPEP §608.01 merely indicates that a web address cannot be incorporated into the specification by reference, and cannot be browser executable. Specifically, the MPEP states:

> Where the hyperlinks and/or other forms of browser-executable codes themselves rather than the contents of the site to which the hyperlinks are directed are part of the applicant's invention and it is necessary to have them included in the patent application in order to comply with the requirements of 35 U.S.C. 112, first paragraph, and applicant does not intend to have these hyperlinks be active links, ***the examiners should not object to these hyperlinks.*** The Office will disable these hyperlinks when preparing the text to be loaded onto the USPTO web database. (emphasis added)

In the present case, the web addresses are not incorporated by reference, and are not active. Thus, deletion of the hyperlinks should not be required.

(2) At item 6 of the Office Action, the Examiner objects to claims 37, 50 and 56 for containing non-elected subject matter.

Non-elected subject matter has been canceled as required.

IV. Response to Claim Rejections Under 35 U.S.C. §112, Second Paragraph

At item 7 of the Office Action, claims 49 and 50 are rejected under 35 USC §112, second paragraph, as being indefinite.

Specifically, the Examiner states that the claims are incomplete for omitting essential steps, such as: a control measurement of IFN-γ, a step of comparing the control measurement with the IFN-γ measurement in the presence of the test compound, and a step of relating the results of the assay back to the objective of the preamble.

Claims 49 and 50 have been amended accordingly.

Withdrawal of this rejection is requested.

V. Response to Claim Rejections Under 35 U.S.C. §112, First Paragraph

(1) At item 8 of the Office Action, claim 38 is rejected under 35 U.S.C. 112, first paragraph, as not being enabled by the specification.

Claim 38 has been canceled.

Withdrawal of this rejection is therefore requested.

(2) At item 9 of the Office Action, claims 40-43 are rejected under 35 U.S.C. §112, first paragraph, as not being enabled by the specification.

Specifically, with respect to claim 40, the Examiner states that the specification does not enable use of SEQ ID NO:188 as a pharmaceutical.

Claim 40 has been amended to recite "a composition."

In addition, submitted herewith is a copy of Shichijo, et al., Two Proliferation-Related Proteins, TYMS and PGK1, Could Be New Cytotoxic T Lymphocyte-Directed Tumor-

Associated Antigens of HLA-A2+ Colon Cancer, *Clinical Cancer Research*, 10:5828-5836 (2004). Shichijo et al. show that the peptide of SEQ ID NO: 188 activates tumor specific CTLs *in vitro*. Specifically, the peptide of present SEQ ID NO: 188 is peptide "189" of "TYMS" (Table 4A of Shichijo), which is shown in Table 5B as being recognized by PBMCs.

With respect to claims 41-43, the Examiner states that the specification does not show that the peptide of SEQ ID NO:188 would be effective as a cancer vaccine or for the treatment of cancer.

Claims 41 and 42 are canceled.

Claim 43 has been amended to recite contacting PBMCs with the peptide of SEQ ID NO: 188 "in vitro."

As discussed above, Shichijo et al. show that the peptide of SEQ ID NO: 188 activates tumor specific CTLs *in vitro*. Specifically, the peptide of present SEQ ID NO: 188 is peptide "189" of "TYMS" (Table 4A of Shichijo), which is shown in Table 5B as being recognized by PBMCs.

Withdrawal of this rejection is requested.

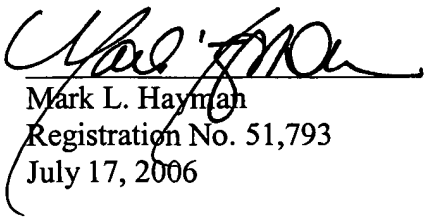
VI. Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order.

If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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Two Proliferation-Related Proteins, TYMS and PGK1, Could Be New Cytotoxic T Lymphocyte-Directed Tumor-Associated Antigens of HLA-A2$^+$ Colon Cancer

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ABSTRACT

Purpose: The purpose of this work was to provide a scientific basis for specific immunotherapy of colon cancer.

Experimental Design: This study focused on identification of colon tumor-associated antigens and HLA-A2–restricted and tumor-reactive cytotoxic T lymphocytes (CTLs) generated from tumor-infiltrating lymphocytes of a colon cancer patient. A gene expression cloning method was used to identify genes coding for tumor antigens. Fifty-six peptides with HLA-A2–binding motifs encoded by these proteins were examined for their ability to induce HLA-A2–restricted and tumor-reactive CTLs.

Results: We identified the following three genes coding for proliferation-related proteins: thymidylate synthase (*TYMS*), which is involved in chemoresistance (5-fluorouracil); 5′-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide transformylase/inosinicase (*AICRT/I*); and phosphoglycerate kinase 1 (*PKGI*), which was secreted by tumor cells and involved in the angiogenic process. *TYMS* was preferentially expressed in tumor cells, whereas *AICRT/I* and *PKGI* were equally expressed in both cancer cells and normal tissues at the mRNA level. Among 56 peptides with HLA-A2–binding motifs encoded by these proteins, 8 peptides were recognized by the CTLs, and 5 of 8 peptides were also recognized by the CTL precursors without *ex vivo* activation in the peripheral blood of colon cancer patients. Furthermore, four of them (one each from TYMS and PKG1 and two from AICRT/1) possessed the ability to induce HLA-A2–restricted and peptide-specific CTLs cytotoxic to colon tumor cells in peripheral blood mononuclear cells of colon cancer patients.

Conclusions: TYMS and PGK1, as well as their epitope peptides, might be appropriate target molecules for specific immunotherapy of HLA-A2$^+$ colon cancer patients because of the positive role of TYMS and PGK1 in chemoresistance (5-fluorouracil) and angiogenesis of tumor cells, respectively.

INTRODUCTION

Colorectal cancer is one of the most commonly occurring malignancies in the world, and the prognosis for patients with advanced colorectal cancer with distant metastasis is extremely poor, despite recent clinical trials with chemotherapeutic agents (1). There is thus a need for the development of new treatment modalities, possibly involving specific immunotherapy. Determination of genes encoding tumor-associated antigens is pivotal for development of a specific immunotherapy. To our knowledge, however, there have been only a few studies that attempted to identify genes encoding tumor-associated antigens in colon cancer. We reported 16 genes encoding tumor-associated antigens by cloning from cDNAs of epithelial cancer cells other than colon cancer cells (2–8), and some of the peptides from these antigens were used as peptide vaccines for colon cancer patients, but no major tumor regression was obtained in this initial clinical trial (9). The other proliferation-related antigens, including breast (HER2/neu) and colon cancer (carcinoembryonic antigen) antigens, were reported to have cytotoxic T lymphocyte (CTL)-directed epitopes (10, 11). However, as far as we know, tumor regression was also not obtained in the clinical trials with those peptides. In the present study, we attempted to determine the molecular features of colon cancer antigens and CTL-directed peptides, and we report herein three proliferation-related proteins and four peptides applicable for use in specific immunotherapy of HLA-A2$^+$ colon cancer.

MATERIALS AND METHODS

Cytotoxic T Lymphocyte Line and Tumor Cell Lines. The parental HLA-A2–restricted and tumor-reactive OK-CTL line was established from tumor-infiltrating lymphocytes (TILs) of a patient with colon cancer (HLA-A0207/3101, HLA-B46/51, HLA-Cw1), as reported previously (8). In brief, TILs were cultured with 45% AIM-V (Invitrogen, Carlsbad, CA) and 45% RPMI 1640 (Invitrogen), 10% fetal calf serum (FCS; MultiSerTM; Trace Scientific Ltd., Melbourne, Australia), 0.1

Received 2/24/04; revised 5/14/04; accepted 5/17/04.
Grant support: Grant-in-Aid 12213134 from the Ministry of Education, Science, Sports, and Culture of Japan (K. Itoh); Grant-in-Aid from the Research Center of Innovative Cancer Therapy of the 21st Century COE Program for Medical Science (K. Itoh and S. Shichijo); Grant-in-Aid 14570526 from the Japan Society for the Promotion of Science (S. Shichijo); and Grants-in-Aid H14-trans-002, 11-16, and H12-cancer-004 (K. Itoh) and H14-cancer-033 (S. Shichijo) from the Ministry of Health and Welfare of Japan.
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mmol/L minimal essential medium nonessential amino acids solution (Invitrogen), and interleukin (IL)-2 (100 units/mL) for more than 50 days. The OK-CTL line with an 80% $CD3^+CD4^-CD8^+$ phenotype showed both HLA-A2–restricted and tumor-specific CTL activity as measured by both chromium-51 release and interferon (IFN)-γ production assays (8). The tumor cell lines used in this study were HLA-A0201$^+$ SW620 colon adenocarcinoma cells, HLA-A0206$^+$ KE3 esophageal squamous cell carcinoma (SCC) cells, HLA-A0207$^+$ Ca9-22 oral SCC cells, HLA-A2$^-$ tumor cells (COLO320 and RERF-LC-MS), autologous Epstein-Barr virus (EBV)-transformed B cells, and phytohemagglutinin P (PHA; Becton Dickinson and Company, Sparks, MD)-activated T cells. One subline of this OK-CTL line, OK-CTLe, was also generated by incubation of OK-CTL with 100 units/mL IL-2 and 10 μg/mL PHA in the presence of irradiated (45 Gy) allogenic peripheral blood mononuclear cells (PBMCs; 2×10^5 cells/well) donated from three HLA-A2$^+$ healthy volunteers as feeder cells. The CTL line was then used as effector cells in the following experiments. SW620 tumor cells were used for preparation of the cDNA library. COS-7 cells or T2 cells were used as the target of a gene expression cloning method or for pulsing peptides, as reported previously (8).

Identification of cDNA Clones and Analyses. A previously reported gene expression cloning method (2, 8) was used to identify genes coding for tumor antigens recognized by the OK-CTLe subline. In brief, poly(A)$^+$ RNA of the SW620 colon tumor cells was converted to cDNA, ligated to the *SalI* adapter, and inserted into the expression vector pCMV-SPORT-2 (Invitrogen). The cDNA clone of *HLA-A0207*, *HLA-A2601*, or *HLA-A2402* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) and inserted into the eukaryotic expression vector pCR3 (Invitrogen). Both 200 ng of plasmid DNA pools or clones of the SW620 library and 200 ng of the *HLA-A0207* cDNA were mixed in 100 μL of Opti-MEM (Invitrogen) with 0.5% of LipofectAMINE (Invitrogen) for 30 minutes. A 50-μL aliquot of the mixture was then added to the COS-7 cells (1×10^3), which were then incubated for 6 hours. RPMI 1640 containing 10% FCS was then added and cultured for 2 days, followed by the addition of the OK-CTLe cells (5×10^3); OK-CTLe is a subline of the parental OK-CTL line. After an 18-hour incubation, 100 μL of supernatant were collected to measure IFN-γ by enzyme-linked immunosorbent assay (ELISA) in a duplicate assay. DNA sequencing was performed with a dideoxynucleotide sequencing method by using a DNA Sequencing kit (Perkin-Elmer, Foster, CA) and analyzed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer). The homology of the sequences of cloned genes derived from the SW620 cDNA library was analyzed using databases on the European Molecular Biology Laboratory GenBank/DDBJ homepage.

For the study of mRNA expression by RT-PCR, total cellular RNA was isolated from colon cancer (COLO201, COLO205, COLO320, SW620, SW480, HCT116, and KM12LM), lung cancer (QG56, RERF-LC-AI, 11-18, and LK87), head and neck SCC (Kuma-1), liver cancer (KIM-1 and KMCH-2), pancreatic cancer (Panc-1), brain tumor (KINGS-1), and leukemia (RAJI) cell lines, and PHA-blast cells and PBMCs were obtained from healthy donors (HDs) by the RNA-Bee RNA isolation reagent (Tel-Test, Inc., Friendswood, TX) method according to the manufacturer's instructions. Total RNAs from normal tissues (spleen, placenta, small intestine, heart, muscle, stomach, lung, liver, brain, testis, kidney, and colon) were purchased from Sawaday Technology (Tokyo, Japan). Complementary DNA of mRNA was prepared from 2 μg of total RNA using a SuperScript Preamplification System (Invitrogen) according to the manufacturer's instructions. Amplification was performed for 25 cycles (1 minute at 94°C, 2 minutes at the annealing temperature for each pair of primer sets, and 3 minutes at 72°C; the annealing temperatures for *SW#029*, *SW#086*, *SW#110*, and β-*actin* were 58°C, 62°C, 60°C, and 58°C, respectively). The separated band corresponding to each of the amplified mRNAs was analyzed by National Institutes of Health Image 1.55f software and integrated to calculate the area. The expression of each mRNA in SW620 tumor cells from which either of the two cDNAs was cloned was defined as 100, respectively. The formula is as follows: percentage of expression of mRNA = (mRNA density of samples/β-*actin* density of sample) × (β-*actin* density of SW620/mRNA density of SW620) × 100.

Peptides and Cytotoxic T Lymphocyte Assay. The only difference in the peptide sequence between HLA-A0201 and HLA-A0207 was the 123rd amino acid, which was Tyr or Cys, respectively. This amino acid was localized in the coil region in the second structure but not in the α-helix or β-sheet, which were involved in peptide binding, and thus this difference might not influence the binding of the peptide. Consequently, in an effort to identify peptides capable of binding to the HLA-A0207 molecules, an internet search was performed for peptides deriving from the thymidylate synthase (TYMS), PGK1 antigens with HLA-A0201–binding motifs (12), and 56 different peptides (>70% purity) were synthesized for screening. To identify CTL-directed epitopes, the CTLs were incubated for 18 hours with T2 cells prepulsed with each peptide at different doses for 2 hours followed by harvesting of supernatant to measure IFN-γ by ELISA. Eight peptides with >90% purity were thereby obtained for the CTL induction experiments. To inhibit IFN-γ production, 20 μg/mL anti-HLA class I (W6/32), anti-HLA class II (H-DR-1), anti-CD4 (Nu-Th/I), anti-CD8 (Nu-Ts/c), and anti-HLA-A2 (BB7.2) was used. Anti-CD14 (JML-H14, IgG2a) served as an isotype-matched control monoclonal antibody (mAb). A two-tailed Student's *t* test was used for the statistical analysis. PBMCs were obtained from the HLA-A2$^+$ cancer patients and HDs and used for CTL induction by the methods reported previously (8). In brief, PBMCs were incubated for 15 to 25 days with 45% AIM-V and 45% RPMI 1640 with 10% FCS and 100 units/mL IL-2 at concentrations of 2 to 4×10^5 cells/well. These activated PBMCs contained 20% to 35% $CD4^-CD8^+$ T cells, and the other cells were mostly $CD4^+CD8^-$ T cells. The IFN-γ production of these activated PBMCs in response to peptide was measured by ELISA.

RESULTS

Identification of cDNA Clones. An HLA-A2–restricted and tumor-specific CTL line was established from the TILs of a patient with colon adenocarcinoma, as reported previously (8). One of the sublines (OK-CTLe) with an 80% $CD3^+CD4^-CD8^+$ phenotype showed HLA-A2–restricted and tumor-specific CTL

activity as measured by IFN-γ production assay (data not shown) and ^{51}Cr release assay (Fig. 1). Namely, OK-CTLe showed cytotoxicity against tumor cells with different HLA-A2 subtypes (HLA-A0201, HLA-A0206, and HLA-A0207), but not to any of the HLA-A2$^-$ tumor cells, autologous PHA-blasts, autologous EBV-transformed B cells, or COS-7 fibroblast cells. Using this OK-CTLe line, 1,000 pools of 100–150 cDNA clones (a total of 1–1.5 × 10^5 cDNA clones) from the cDNA library of SW620 tumor cells were screened by the gene expression cloning method followed by selection to identify specific cDNAs within positive pools. After repeated experiments, we identified the following three known genes encoding for proliferation-related proteins. Complementary DNA *SW#029* (accession number AB062290) encoded *Homo sapiens* TYMS (accession number: XM008753), which is considered to be a measure of 5-fluorouracil (5-FU) chemosensitivity for colorectal carcinoma (13). Complementary DNA *SW#086* (accession number AB062403) encoded *H. sapiens* 5′-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide transfolmylase/inosinicase (AICRT/I; accession number D82348). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transfolmylase-inoshin monophosphate cyclohydrolase (ATIC), the product of the *purH* gene, is a bifunctional enzyme that catalyzes the two activities in *de novo* purine biosynthesis, AICAR transformylase and inosone 5′-monophosphate (IMP) cyclohydrolase (14). Complementary DNA *SW#110* (accession number AB062432) encoded the *H. sapiens* phosphoglycerate kinase 1 (PGK1; accession number XM010102). PGK influences DNA replication and repair in mammalian cell nuclei (15–17). It is also secreted by tumor cells and participates in tumor angiogenesis (18). Representative results of the CTL assays are shown in Fig. 2. This OK-CTLe subline did not react to COS-7 cells cotransfected with

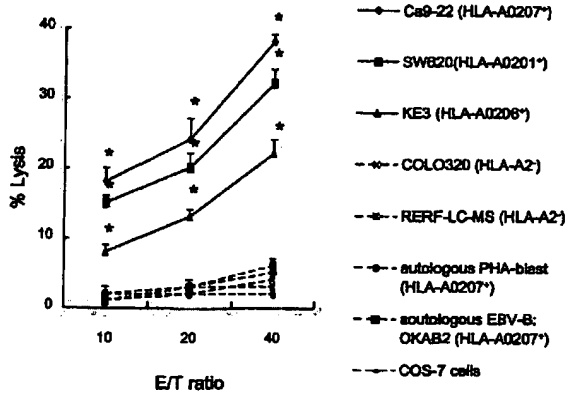

Fig. 1 Characteristics of HLA-A2–restricted OK-CTLe. The cytotoxicity of OK-CTLe against various target cells was tested by a 6-hour ^{51}Cr release assay at different effector to target cell ratios in triplicate assays. Target cells were HLA-A0207$^+$ Ca9-22 head and neck SCC, HLA-A0201$^+$ SW620 colon adenocarcinoma, HLA-A0206$^+$ KE3 esophageal SCC, HLA-A2402$^+$ COLO320 colon adenocarcinoma, and HLA-A1101$^+$ RERF-LC-MS lung adenocarcinoma cell lines; autologous PHA-blastoid cells; autologous EBV-transformed B cells (OKAB2); and COS-7 cells. Values represent the mean of percentage of specific lysis of triplicate determinations. A two-tailed Student's *t* test was used for the statistical analysis (*, $P < 0.05$) against a negative control (COLO320, HLA-A2$^-$ cell line).

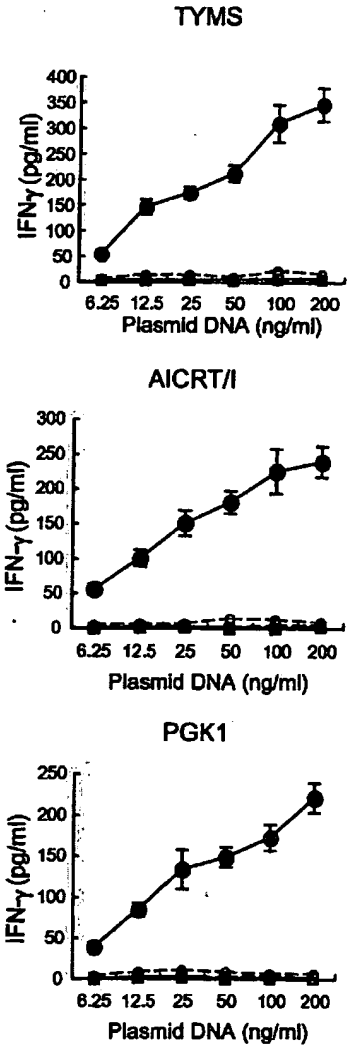

Fig. 2 Genes coding for tumor epitopes. Different amounts of each of three cDNA clones derived from SW620 tumor cells and 100 ng of *HLA-A0207*, *HLA-A2402*, or *HLA-A2601* cDNA were cotransfected into COS-7 cells, incubated for 48 hours, and then tested for their ability to stimulate IFN-γ release by the OK-CTLe lines. The background of IFN-γ release by the CTLs in response to COS-7 cells (under 50 pg/mL) was subtracted from the values in the figure. Complementary DNA *clone #001* represents the irrelevant clones cotransfected with HLA-A0207 (○); cloned cDNA with HLA-A2601 (□) or cloned cDNA alone (△) was not recognized by the OK-CTLs. Values represent the means of triplicate assays of the data from cDNA cotransfected with *HLA-A0207* (●), which values were significantly higher than those for the negative control cDNA *clone #001* at two or more different concentrations of plasmid DNAs. A two-tailed Student's *t* test was used.

HLA-A0207 cDNA and cDNA *clone #001* taken as a negative control (Fig. 2, ○). This subline also failed to react to COS-7 cells transfected with any of the three cDNA clones (Fig. 2, △) or cotransfected with any of these three cDNA clones and *HLA-A2601* cDNA as the other negative controls (Fig. 2, □).

Gene Expression Levels. The mRNA expression of these genes was investigated by the semiquantitative RT-PCR method using 17 tumor cell lines and 14 normal cells or tissues (Fig. 3). *TYMS* was highly expressed in the majority of tumor cells, with a few exceptions, whereas *TYMS* expression was very low in the majority of normal tissues with a few exceptions (testes, placenta, and small intestine). In contrast, *AICRT/I* and *PGK1* were equally expressed in both tumor cells and normal tissues.

Determination of Cytotoxic T Lymphocyte Epitopes. To determine CTL-directed epitopes, each of the 56 different synthesized peptides with HLA-A2 molecule-binding motifs derived from these proteins or a human immunodeficiency virus (HIV)-derived peptide with an HLA-A2-binding motif as a negative control was loaded onto T2 cells followed by testing for their ability to stimulate IFN-γ release by OK-CTLe. Eight peptides (three from TYMS, three from AICRT/I, and two from PGK1) among them were recognized by OK-CTLe (Fig. 4*A*). The peptide sequence and position of the first amino acid of the peptides are shown on the *left* of the column. In TYMS, AICR/I, and $PGK1_{222}$ cases, the amounts of IFN-γ increased in a peptide concentration-dependent manner. On the other hand, the ability to stimulate IFN-γ production was observed at the maximal level with 1 μg/mL $PGK1_{235}$ (Fig. 4*B*). The high avidity CTL capable to recognize at low-dose peptide-pulsed target cells, and these cells sometime suppressed at higher-dose of peptides.

Cytotoxic T Lymphocyte Precursors in the Circulation. We also investigated whether CTL precursors reactive to these antigens would be detectable in PBMCs of HLA-A2$^+$ colon cancer patients. PBMCs from three patients [a colon cancer patient (OK) from whose tumor the parental CTL line was obtained, the other colon cancer patient (TT, HLA-A0201), and a metastatic melanoma patient (KM, HLA-A0201) as a negative control] and an HLA-A2$^+$ HD (HLA-A0201) as the other negative control were expanded *in vitro* with IL-2 alone for 15 to 25 days and then tested for their reactivity to each of these three cDNA clones. None of the clones was recognized by the activated PBMCs of a melanoma patient or a HD (data not shown). However, the activated OK PBMCs recognized *AICRT/I* and *PGK1*, and furthermore, those of the colon cancer patient (TT) recognized *AICRT/I* (Fig. 5*A*).

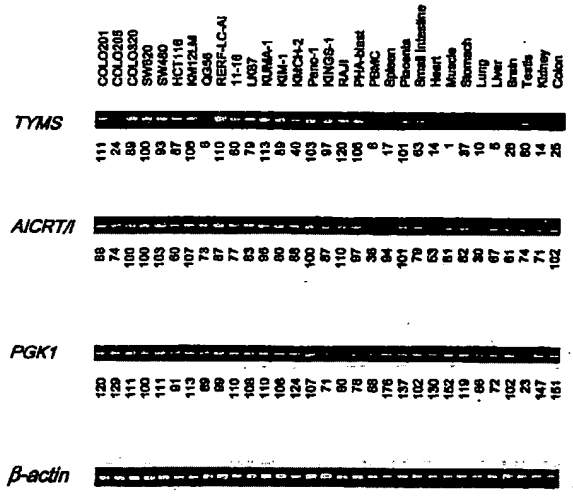

Fig. 3 Messenger RNA expression by semiquantitative RT-PCR. The relative expression of mRNA is shown as the ratio of the density of each sample to the β-*actin* density of RT-PCR products. The primer pairs were as follows: 029-186S, 5′-GCAGATCCAACACATCCTCC-3′; 029-795AS, 5′-CGCAATCATGTACGTGAGC-3′; 086-329S, 5′-GTTGCCTGCAATCTCTATCCC-3′; 086-798AS, 5′-CCTTGAGTTCCTTCACCAGC-3′; 110-194S, 5′-AGAGGATTAAGGCTGCTGTCC-3′; 110-838AS, 5′-TCTCCATGTTGTTGAGCACC-3′; β-*actin*-60S, 5′-CTTCGCGGGCGACGATGC-3′; and β-*actin*-381AS, 5′-CGTACATGGCTGGGGTGTTG-3′.

Each of the 56 peptides described above was then tested for its ability to stimulate IFN-γ production by PBMCs incubated with IL-2 (100 units/mL) alone. These IL-2–activated PBMCs from either a melanoma patient or a HD did not react to any peptides directed at the three gene products tested (data not shown). In contrast, the IL-2–activated OK PBMCs reacted to three (one from each of the three proteins) of the eight peptides that were also recognized by the parental CTL line (Fig. 5*B*). Furthermore, the activated PBMCs of the TT case also recognized three (one from TYMS and two from AICRT/I) of these eight peptides (Fig. 5*B*). IFN-γ production by these cells in response to peptides was inhibited by 20 μg/mL anti-HLA class I, anti-CD8, or anti-HLA-A2 mAb, but not by anti-HLA class II, anti-CD4, or anti-CD14 serving as an isotype-matched control mAb (data not shown). These results suggest that CTL precursors reactive to at least one peptide from each of these three proliferation-related proteins are detectable in the circulation of colon cancer patients without *ex vivo* stimulation by the corresponding peptides.

Cytotoxic T Lymphocyte Induction by Peptides. Based on these results, these eight peptides were tested for their ability to induce CTL activity in PBMCs of 10 HLA-A2$^+$ colon cancer patients (subtypes: 4 HLA-A0201, 3 HLA-A0206, and 3 HLA-A0207). PBMCs were stimulated *in vitro* with each of the eight peptides (10 μg/mL) and IL-2 (100 units/mL) every 3 days for 15 days, followed by incubation with IL-2 alone for an additional 15 days, and tested for their cytotoxicity in triplicate experiments by a 6-hour ^{51}Cr release assay against HLA-A2$^+$ SW620 tumor cells in the presence or absence of various mAbs. HLA-A2$^-$ QG56 tumor cells and HLA-A2$^+$ PHA-blast cells were used as negative controls for the ^{51}Cr release assay. The peptide specificity of the cytotoxicity was also confirmed by competition assay by the addition of excess numbers of T2 cells pulsed with a corresponding peptide or a HIV peptide as a negative control. Among the eight peptides tested, AICRT/I at positions 90–98, PGK1-derived peptide at positions 235–243, TYMS at positions 189–198, AICRT/I at positions 288–296, and TYMS at positions 231–240 induced HLA-A2–restricted and peptide-specific cytotoxicity against SW620 tumor cells in the PBMCs from 6, 5, 4, 4, and 1 of 10 patients irrespective of the different HLA-A2 subtypes, respectively (Fig. 6*A* and *B*). The levels of cytotoxicity of the peptide-stimulated PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by the other mAbs tested in the assay. The cytotoxicity was also inhibited by the addition of the corresponding peptide-pulsed T2 cells, but not by that of the HIV peptide-pulsed cells in all of the cases tested. These results

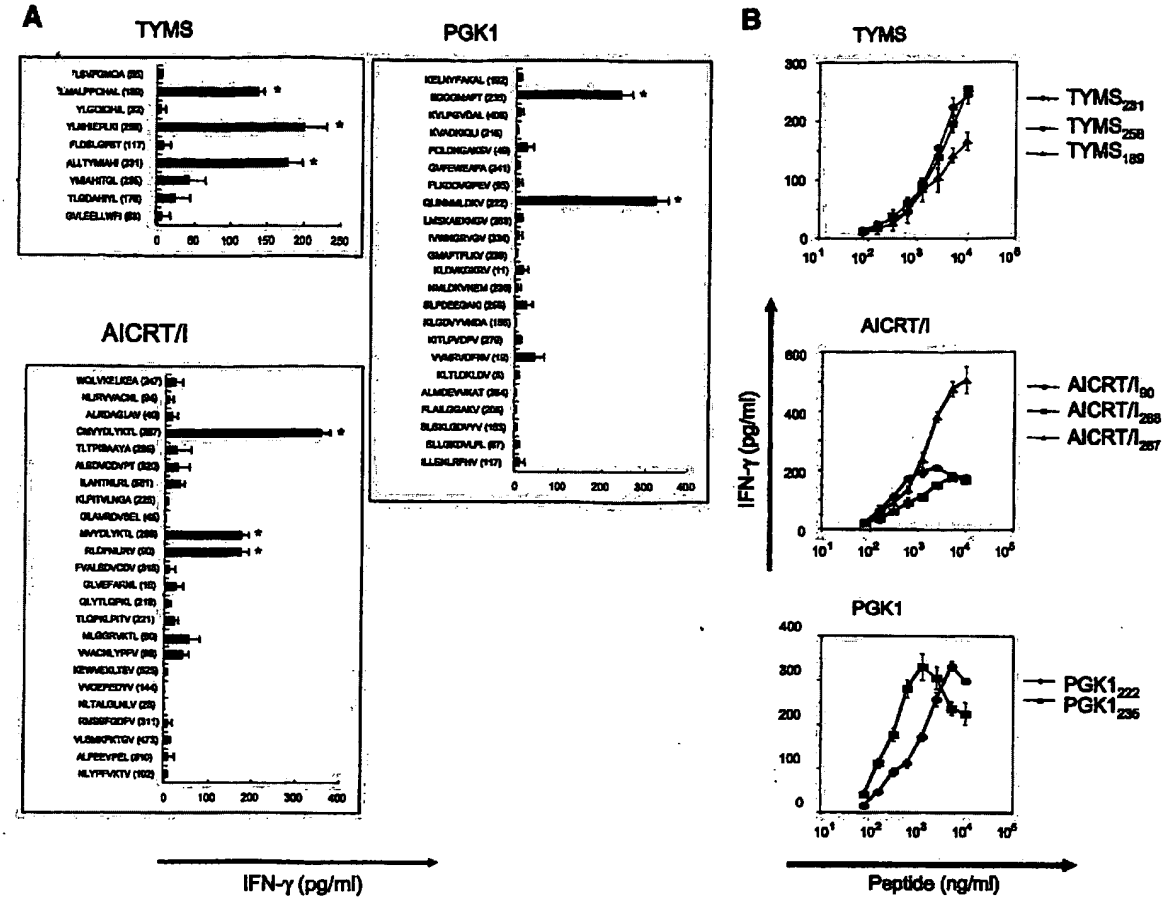

Fig. 4 Determination of epitopes. *A*. Each of the peptides (9–10-mer) or a HIV-derived peptide with an HLA-A2–binding motif as a negative control was loaded onto T2 cells at various concentrations of peptides for 2 hours. The OK-CTLe cells were then added and incubated for 18 hours, followed by collection of cell-free supernatant for measurement of IFN-γ. Values are the means of triplicate assays. The background of IFN-γ release by the CTLs (<50 pg/mL) in response to the T2 cells alone was subtracted from the values in the figure. Peptides on T2 cells that could stimulate a significant amount of IFN-γ production are indicated (*, $P < 0.05$ by two-tailed Student's *t* test and 100 pg/mL > IFN-γ). *B*. In all cases, the amount of IFN-γ increased in a peptide concentration-dependent manner. Representative results are shown in the figure. Values are the mean of triplicate assays.

suggest that the CTL activity induced by each of these four peptides was largely mediated by the peptide-specific and tumor-reactive $CD8^+$ T cells in an HLA class I-restricted manner. In contrast, the remaining three peptides induced such CTL activity in the PBMCs from any patient tested (data not shown).

DISCUSSION

In this study we reported three proliferation-related proteins as new CTL-directed tumor-associated antigens that were recognized by the CTL line from T cells infiltrating into colon tumor. These cell proliferation-related proteins would be vigorously synthesized, used, and then processed in tumor cells. Consequently, the processed peptides might be loaded onto major histocompatibility complex class I molecules at higher levels than those of immunologic ignorance but still at levels lower than those to induce immunologic tolerance. In this case, a few proteins involved in proliferation might act as colon tumor-associated antigens recognized by CTLs. Thus, there might be relatively low but distinct adaptive immunity against autologous colon tumor cells.

Colon tumor cells often become resistant to 5-FU–based chemotherapy. The up-regulated expression of TYMS, one of the antigens identified in this study, might be considered to be a parameter of 5-FU chemosensitivity for colorectal carcinoma (13). Indeed, the chemosensitivity of 5-FU for colorectal carcinomas with a low expression of TYMS has been reported to be better than that for tumors showing high expression of TYMS. Overall survival is reported to be better in the TYMS−(−) group than in TYMS−(+) group. Recent systemic review and meta-analysis provided evidence that colorectal tumors expressing high levels of TYMS appeared to have a poor overall survival compared with tumors expressing low levels of TYMS (15). Uchida *et al.* (16) also provide further support to the growing body of evidence that TYMS expression may be used

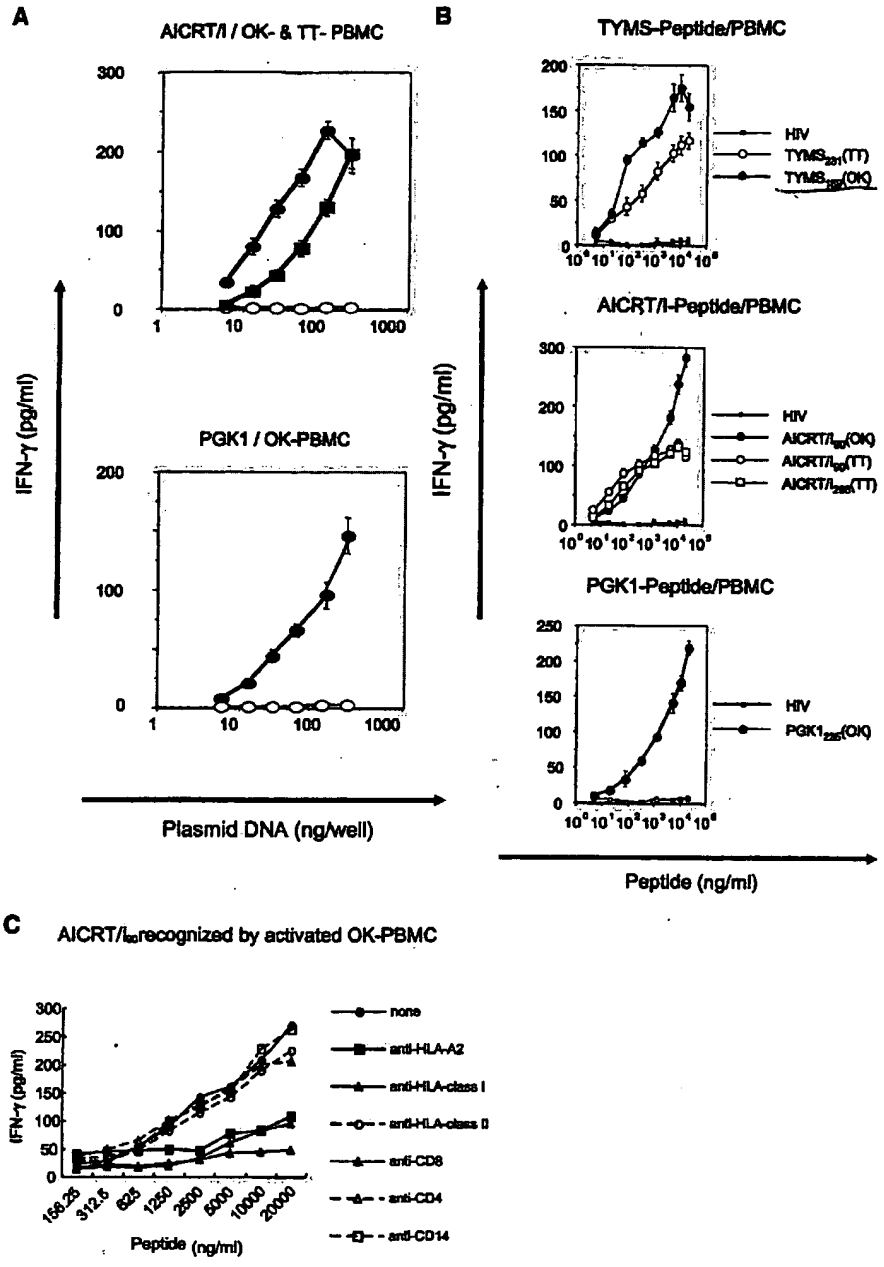

Fig. 5 Recognition of gene products and peptides by the PBMCs. *A*. Different amounts of each of the three cDNA clones and 100 ng of *HLA-A0207* or *HLA-A02402* cDNA were cotransfected into COS-7 cells, followed by a test of their ability to stimulate IFN-γ release by the activated OK-PBMCs (●) or TT PBMCs (■). Results for cDNA clones and HLA-A0207 are shown. Complementary DNA *clone #001* represents the irrelevant clones that were not recognized by the OK-CTLs (○). Values represent the means of triplicate assays of the data from cDNA cotransfected with *HLA-A0207* (●), which values were significantly higher than those for the negative control cDNA *clone #001* at two or more different concentrations of plasmid DNAs. A two-tailed Student's *t* test was used. *B*. Recognition of peptides by the activated OK PBMCs was tested. Each of the peptides or a HIV-derived peptide with an HLA-A2–binding motif as negative control was loaded onto T2 cells at various concentrations of peptides for 2 hours. The activated PBMCs were then added and incubated for 18 hours, followed by collection of cell-free supernatant for measurement of IFN-γ. Values represent the means of triplicate assays. The ability to stimulate IFN-γ production by the activated OK PBMCs (*closed symbols*) or TT PBMCs (*open symbols*) was assayed by ELISA. *C*, inhibition of IFN-γ production by mAbs. A representative result for AICRT/I_{90} is shown. AICRT/I_{90}-pulsed T2 cells were used as target cells for activated OK PBMCs (2×10^5 cells). The methods for preparation of peptide-pulsed T2 cells and inhibition assay are described in Materials and Methods.

as a molecular biomarker of response to TYMS-directed chemotherapy. PGK is known as the sixth enzyme of the glycolytic pathway, in which it equilibrates transfer between position 1 of 1,3-bisphosphoglycerate and the γ-phosphate of $MgATP^{2-}$. PGK also influences DNA replication and repair in mammalian cell nuclei (17, 18). The hypoxic nature of solid tumors triggers expression of vascular endothelial cell growth factor, which in turn stimulates both angiogenesis and glycolytic enzyme activity, including that of PGK, with the ability to facilitate anaerobic production of ATP (19). PGK not only functions in glycolysis but also is secreted by tumor cells and participates in the angiogenic process as a disulfide reductase (20). All these results suggest that both TYMS and PKG1 proteins are appropriate for use as CTL-directed tumor antigens because of their possible roles in tumor growth, whereas the involvement of AICRT/I protein in proliferation of colon cancer cells remains uncertain.

It is well known that colonic epithelial stem cells vigor-

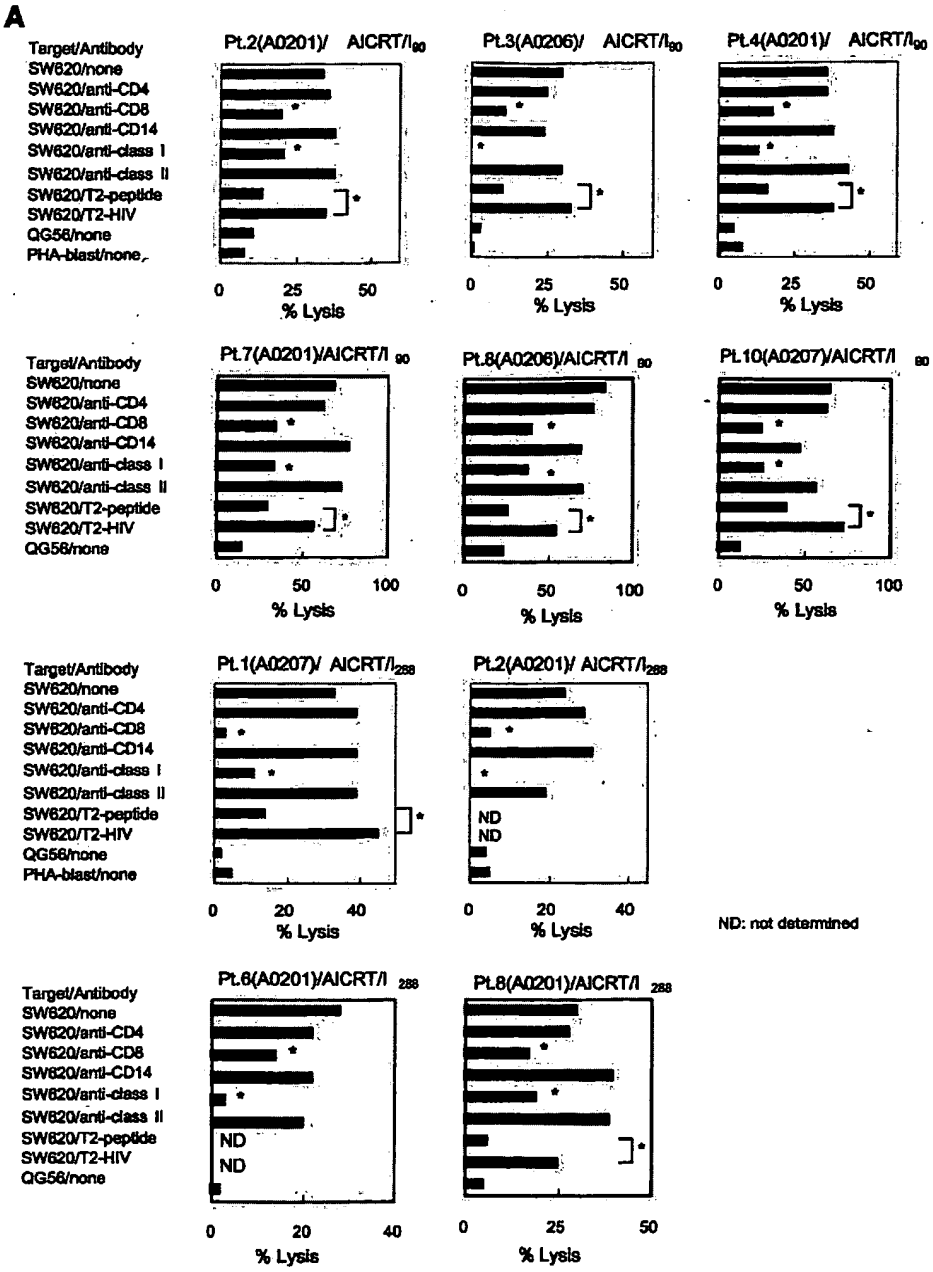

Fig. 6 Cytotoxicity by the peptide-stimulated PBMCs. Each of these eight peptides was tested for its ability to induce CTL activity in PBMCs of five HLA-A2$^+$ colon cancer patients. Their HLA-A2 genotypes were determined by a previously reported method (10); patients 2 and 4 had the A0201 genotype; patients 3 and 5 had the A0206 genotype; and patient 1 had the A0207 genotype. PBMCs were stimulated with each of the eight peptides (10 μg/mL) and IL-2 (100 units/mL) every 3 days for 15 days, followed by incubation with IL-2 alone for an additional 15 days, and tested for their cytotoxicity against HLA-A2$^+$ SW620 tumor cells in the presence or absence of various mAbs by a standard 6-hour ^{51}Cr release assay at an effector to target cell ratio of 10:1 in triplicate assays. HLA-A2$^-$ QG56 tumor cells and HLA-A24$^+$ PHA-blast cells were used as negative controls for the ^{51}Cr release assay. The peptide specificity of the cytotoxicity was also confirmed by competition assay by addition of T2 cells pulsed with a corresponding peptide or a HIV peptide as a negative control at a hot to cold cell ratio of 20:1 in triplicate assays. Results for the AICR/I peptides (panel A) and for the TYMS or PKG1 (panel) peptides that had the ability to induce CTLs from at least two patients are shown in Fig. 6. A two-tailed Student's *t* test was used for the statistical analysis in this study; *P* values of <0.05 were considered to indicate statistical significance. Values represent the means ± SD of the percentage of specific lysis.

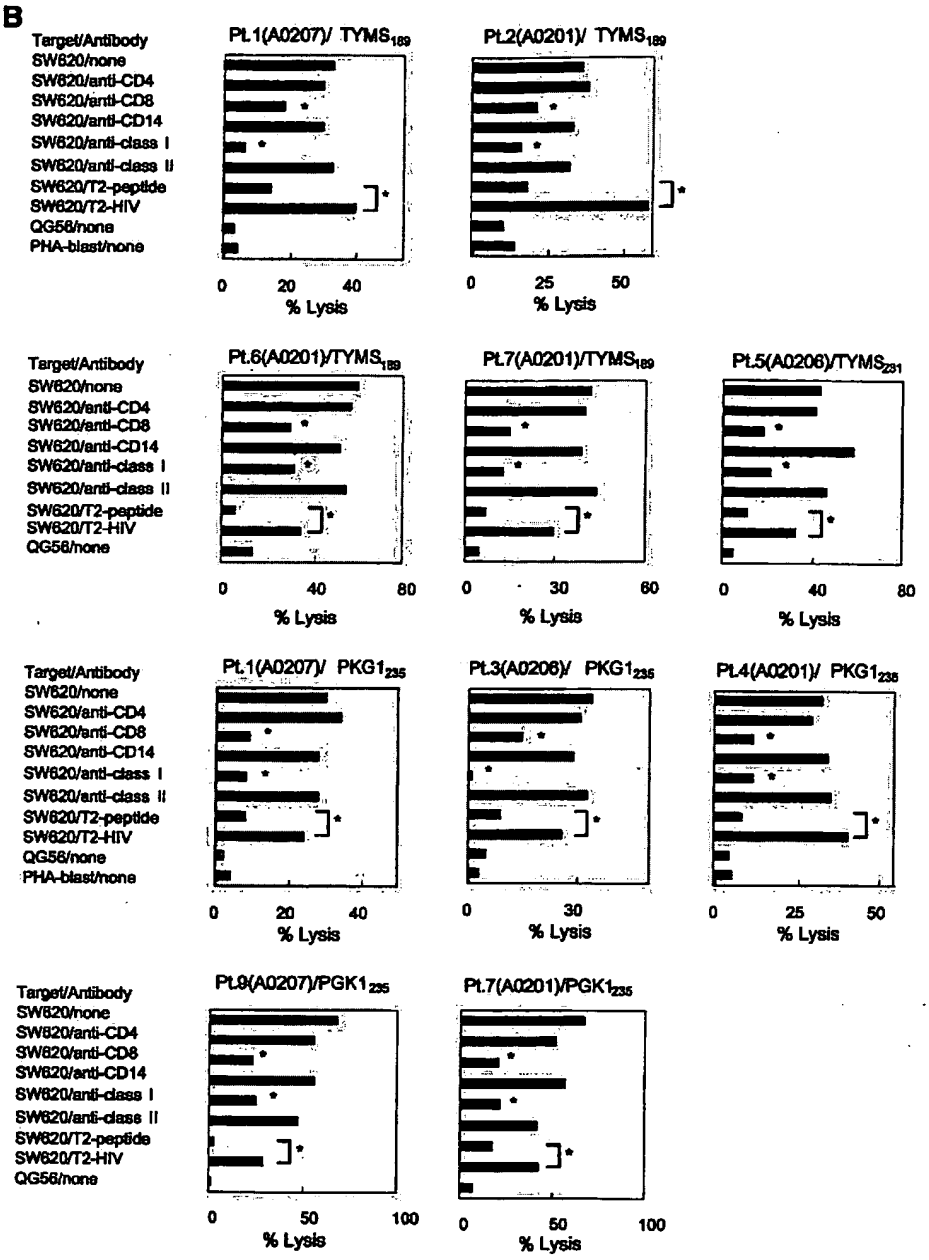

Fig. 6 Continued.

ously proliferate, differentiate, and mature and then quickly turnover via apoptosis. Therefore, adverse events such as diarrhea are expected to be associated with vaccination of peptides derived from proliferation-related proteins. However, no severe adverse effects were observed in our phase I clinical trials of individualized peptide vaccination for advanced cancer patients, which were performed using peptides from proliferation-related proteins (9, 21–25). However, whether or not proliferation-related peptides are suitable as vaccines for the treatment of colon cancer needs to be carefully evaluated through additional basic and clinical studies. It also remains to be elucidated whether or not these CTL epitope peptides derived from proteins ubiquitously expressed in both tumor cells and normal cells at the mRNA level are suitable for use in peptide-based cancer immunotherapy. Among the previously reported CTL-directed antigens, most were ubiquitously expressed in both

tumor cells and normal tissues at the mRNA level, with preferential or exclusive expression in tumor cells at the protein level (2, 4–8), whereas *MRP3* showed preferential expression at the mRNA level (3). However, in our above-mentioned clinical trials of peptide vaccinations for several epithelial cancers, including colon cancer, there were no apparent differences in safety or tumor regression between the peptides from the former group and those from the latter group, and major tumor regression was observed in a few patients with no severe adverse events (21–25).

The HLA-A2 allele is found in 23% of Africans, 53% of Chinese, 40% of Japanese, and 50% of Caucasians (12). With regard to HLA-A2 subtypes, HLA-A0201 is found in the majority of HLA-$A2^+$ Caucasians and in 45% of HLA-$A2^+$ Japanese, whereas HLA-A0206 is found in 36% of HLA-$A2^+$ Japanese (26). PGK1-derived peptide at positions 235 to 243 induced CTL activity in PBMCs from patients with all three HLA-A2 subtypes, whereas AICRT/I at positions 90 to 98 induced CTL activity in PBMCs of HLA-A0201 and HLA-$A0206^+$ patients. Two peptides (TYMS at positions 189 to 198 and AICRT/I at positions 288 to 296) induced CTL activity in HLA-A0201 and HLA-$A0207^+$ patients. These results suggest that each of the four peptides has the ability to induce HLA-A2–restricted CTLs reactive to tumor cells, in agreement with our previous observation (8). However, additional studies with more samples with different subtypes will be needed to confirm whether or not each of these four peptides has the ability to induce HLA-A2–restricted and tumor-reactive CTLs from HLA-A2 colon cancer patients irrespective of the HLA-A2 subtype.

The results of these studies showed that four peptides from three proliferation-related proteins had the ability to induce HLA-A2–restricted CTLs reactive to colon tumor cells in PBMCs of colon cancer patients. These results may provide a better understanding of the molecular features of colon cancer antigens and CTL-directed peptides.

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